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Original Article

Expression and promoter activity of homeodomain factor Nanog in pancreatic cancer cells

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Abstract: NANOG is a transcription factor in embryonic stem cells (ESCs) and thought to be a key factor in maintaining pluripotency. Moreover, Nanog gene expression closely related to the differentiation status of tumor and played an important role in promoting the generation and development of tumor cell, as well as anti apoptosis. So, the purpose of this paper was to clone Nanog gene promoter and dual luciferase reporter vector was constructed to detect the promoter activity. Three different length promoter of Nanog gene was cloned by PCR approach and inserted into pGL3-Basic vector to construct a recombinant vector pGL3-1852, pGL3-1112 and pGL3-753, respectively. Panc-1 cells were transfected by the recombinant vector and the transcriptional activity was determined by the dual luciferase reporter assay system so as to find the core promoter region and its regulation model. Methylation inhibitor (5-aza-2'-deoxycytidine, 5-Azadc) and histone deacetylase inhibitors (Trichostatin A, TSA) was used to induce the transcription of Nanog gene in Bxpc-3 cells. The results showed that three different length fragment of Nanog gene was cloned correctly; the sequence homology is 99% with the sequence published in *Genbank*. Dual luciferase report gene expression vector pGL3-1852, pGL3-1112 and pGL3-753 were obtained. The transcriptional activity of pGL3-1852 was the strongest. The transcription activity of Nanog was enhanced when 5-Azadc and TSA was used, suggesting the possibility of elevated methylation of the CpG island in the Nanog regulatory region in Bxpc-3 cells.

Keywords: Pancreatic cancer cells, homeodomain factors Nanog, dual luciferase reporter gene assay

Introduction

Pancreatic adenocarcinoma is a highly lethal disease, which is usually diagnosed in an advanced state for which there is little or no effective therapies. It has the worst prognosis of any major malignancy (3% 5-year survival) [1]. Along with deep research on the stem cells and the tumor origin, people realized that stem cell's dys-differentiation and the multiplication can cause the occurrence of the tumor. There are very complicated relations between the tumor origin, malignancy, drug resistance, metastasis, recurrence and the unusual expression of stem cell genes. Therefore, the identification and control of tumor stem cells have a great significance for cancer prevention, early detection, diagnosis and treatment.

Human NANOG protein is a 305 amino acid protein with a conserved homeodomain motif that is localized to the nuclear component of cells [2]. The homeodomain region facilitates DNA binding. There are C-terminal, N-terminal and homeodomain regions in human NANOG protein. The latest study showed that Nanog not only expressed in germline stem cell, embryonic germ cell tumors and embryonic stem cell, but also in some cancer cells (breast cancer, germ cell tumors, glioma, germ cell tumor). In view of the cancer cells and embryonic stem cells have infinite proliferation value and keep the characteristic of the low state, suggesting that Nanog may a key factor in regulating cell self-renewal and pluripotency. In addition, knockout or the silence of Nanog gene can induce tumor cell differentiation, also lead to cell cycle arrest and inhibition of tumor development. These results fully demonstrated that Nanog gene expression related closely to the differentiation status of tumor and played an important role in promoting the generation and

development of tumor cell, as well as anti-apoptosis.

Although many studies have demonstrated that Nanog was expressed in the tumor of embryonic, but for the expression of Nanog in solid tumors have not been reported, meanwhile, there is still blank about the relationship between its structure and function, as well as its gene regulation mode. Therefore, three pancreatic cancer cell lines with different differential degrees (Bxpc-3, Pane-1 and Mia PaCa-2) was searched to observe the Nanog gene expression from mRNA and protein level, and the relationship between Nanog gene expression and pancreatic cancer cell differentiation level was preliminarily revealed. Meanwhile, three different length promoter of Nanog gene was cloned by PCR approach and inserted into pGL3-Basic vector to construct a recombinant vector pGL3-1852, pGL3-1112 and pGL3-753, respectively. Panc-1 cells were transfected by the recombinant vector and the transcriptional activity was determined by the dual luciferase reporter assay system so as to find the core promoter region and its regulation model. Finally, to lay the foundation for revealing the regulatory role of Nanog in pancreatic cancer cells and its function in mediating the origin of pancreatic cancer, biological behavior such as invasion and metastasis development.

Materials and methods

Cells and reagent

Three pancreatic cancer cell lines with different differential degrees (Bxpc-3, Pane-1 and Mia PaCa-2) were purchased from ATCC. *E. coli* DH5α competent cells, gel extraction kit and miniprep kits were purchased from Tiangen Biotech (Beijing) Co., Ltd.. DL5000 DNA Marker Prime, STARMax DNA Polymerase, T4 DNA ligase and restriction endonuclease were purchased from Takara Biotechnology (Dalian) Co., Ltd.. Expression vector pGL3.0-Basic, pRL-SV40 and Dual-Luciferase Reporter Assay System were purchased from Promega Corporation. LipofectamineTM2000 was purchased from Invitrogen Corporation. 5-Azadc (5-aza-2'-deoxycytidine) or TSA (Trichostatin A) were purchased from ProSpec-Tany TechnoGene Ltd.. Primer synthesis and sequencing were conducted by the Invitrogen Company of Shanghai.

Plasmid construction

All constructs containing deletions in the Nanog promoter were generated by PCR from genomic DNA using primers containing appropriate restriction sites at the 5' end. The primer sequence was shown as the followed: pGL3-1852 forward 5' cggggtacc ATCGGGATTTGCTAAGAGTT 3', pGL3-1112 forward 5' cggggtacc CTTGGCGAAGAATGTAGTAAG 3', pGL3-753 forward 5' cggggtacc GATCACTGAGGATCGTCCAA 3', they shared the same reverse 5' ccgctcgag CAAGGATGGATAGTTTTCTTCAGGC 3'. The amplified fragments were separated by electrophoresis on a 1% agarose gel, were excised and purified with a High Pure PCR Product Purification Kit (Tiangen Biotech (Beijing) Co., Ltd.) and subsequently sequence-verified. Purified PCR fragments were digested and cloned into the pGL3-Basic reporter construct (Promega). The promoter region of Nanog gene and its potential transcription factor binding sites were predicted and analyzed using online software TFsearch and AliBaba2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). The methylation sites of Nanog gene were predicated by the software (http://www.urogene.org/cgi-bin/methprimer/methprimer_results.cgi).

Cell culture and treatment

Bxpc-3, Pane-1 and Mia PaCa-2 were cultured in Dulbecco's Modified Eagle's medium (high glucose, Sigma) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 100 IU/ml penicillin and 100 μg/ml streptomycin. All cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C and passaged every 2-3 days. Cells were grown to approximately 80% confluency for transfection purposes. Four hours prior to transfection, medium was changed to Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Invitrogen).

DNA transfection and dual luciferase assays

Cells were plated in 24-well dishes and transfected with 1.0 μg/dish firefly luciferase reporter plasmids or pGL3-Basic (Promega), together with 40 ng/well of Renilla luciferase plasmid pRL-SV40 (Promega) as an internal control using LipofectamineTM2000 (Invitrogen). At 24 hours post transfection, medium was changed and the cells were subjected to 5-Azadc or TSA

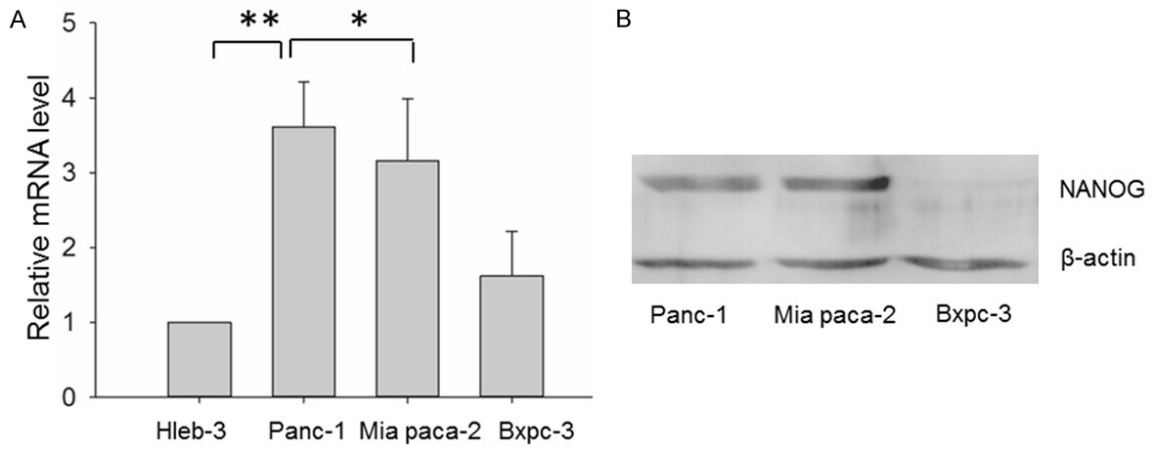

Figure 1. The mRNA and protein expression level was detected by qPCR and Western blotting. A. Three pancreatic cancer cell lines with different differential degrees (Bxpc-3, Pane-1 and Mia PaCa-2) was selected, mRNA levels in those three cell lines were appreciable as measured by quantitative real-time PCR (qPCR). B. Protein level in three cell lines was assessed by Western blotting.

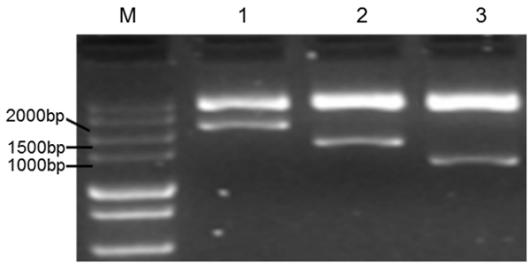

Figure 2. Identification of the pGL3-Nanog by restriction enzyme digestion. M: DNA marker; 1~3: Restriction enzyme digestion products of the pGL3-1852, pGL3-1118 and pGL3-798.

treatment. Cells were harvested and lysed after 48 h treatment, and dual luciferase assays were performed according to manufacturer instructions (Promega). Luciferase activity was measured using Promega GloMax 20/20 and calculated as relative luciferase activity (firefly luciferase/Renilla luciferase) to correct for transfection efficiency. All assays were repeated independently three times, and results are shown with standard errors.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from Bxpc-3, Pane-1 and Mia PaCa-2 cells using TRIZOL reagent (Invitrogen) as per manufacturer's protocol. Quantification of total RNA was done on a Nanodrop 8000 (Thermo Scientific) in addition to purity assessment on a 1% agarose gel. cDNA was synthesized from 5 μg of total RNA using Superscript II (Invitrogen) using the manufacturer's recommended protocol. qPCRs were performed with iTaq SYBR Green/ROX supermix (BioRad) in an ABI 7300 with the thermal profile: initial activation at 95°C for 3 min, 40 cycles of 95°C for 15 and 60°C for 45 sec. The following primer sets were used: Nanog forward (5'-TGAACCTCAGCTACAAACAG-3') and reverse (5'-TGGTGGTAGGAAGAGTAAG-3'), β-actin forward (5'-TGCACCACCAACTGCTTAG-3') and reverse (5'-GGCAGGGATGATGTTC-3'). Standard curves were used to ensure equivalent primer efficiency and melting curves were used to confirm specificity. Ct values were converted to expression values using the ΔΔCt method using GAPDH as the reference gene.

Statistical analysis

Statistical analysis was performed using SPSS version 18.0 (SPSS, Chicago, IL, USA). All data are expressed as the mean ± SD. Differences between groups were evaluated with one-way analysis of variance with a post hoc test (Student-Newman-Keuls method); $P < 0.05$ was considered statistically significant (two-tailed).

Results

Expression level of Nanog in three pancreatic cancer cell lines with different differential degrees

First we screened cell lines available in our laboratory for endogenous levels of Nanog. Three pancreatic cancer cell lines with different dif-

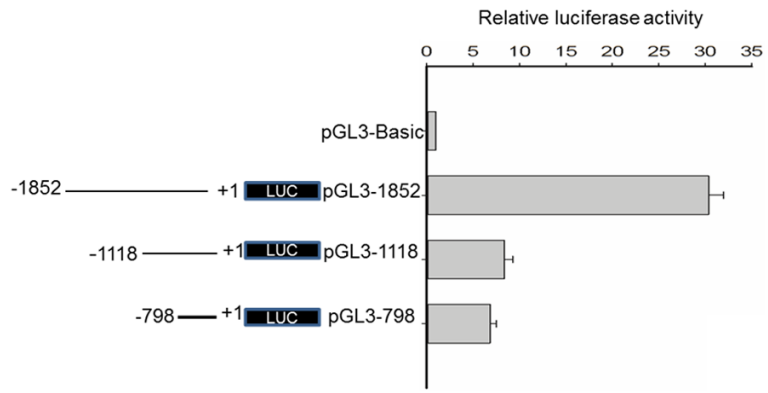

Figure 3. Activity of different promoters of Nanog gene in Panc-1 cells. Panc-1 cells transfected with those three plasmids and the dual luciferase assays was performed, pGL3-Basic was used as the negative control.

ferential degrees (Bxpc-3, Pane-1 and Mia PaCa-2) was selected, human lens epithelial B-3 (HLEB-3) cells was used as the control. mRNA levels in those three cell lines were appreciable as measured by quantitative real-time PCR (qPCR) (**Figure 1A**). Subsequently we chose Pane-1 in our further analyses, on the basis of potentially using Pane-1 as cell models for our future functional studies.

To examine whether NANOG is also expressed in those three cell line, Bxpc-3, Pane-1 and Mia PaCa-2 cells were grown in media for 24 hours and the NANOG protein level was assessed by Western blotting. We found that NANOG was significantly obtained in Pane-1 and Mia PaCa-2 cell line, while was not or low expression in Bxpc-3 cell line (**Figure 1B**).

Molecular cloning of the potential Nanog promoter

To investigate whether Nanog was induced at the transcription level, we sought to capture the functional promoter region in the genome. For that, a series of three promoter fragments from 1.8 kb (-1852 bp to +1) upstream of the transcription start site were amplified by PCR and cloned into pGL3-Basic firefly luciferase reporter plasmid. The promoter fragments in the resulting reporter plasmids shared the same 3' end (+1) with the 5' end beginning at bases -1118, and -798 from the transcription start site. Double enzyme digests with *Kpn* I and *Xho* I was conducted, the size and sequencing results were consistent with the expected results, indicating three promoter fragments plasmid construction, it could be used for the following experiment (**Figure 2**).

Identification of minimal region (s) required for basal promoter activity of Nanog

When we transfected those three plasmids into Panc-1 cells and performed the dual luciferase assays, pGL3-Basic was used as the negative control. The results showed that the reporter activity was induced by these three promoter fragments. Compared with pGL3-Basic, pGL3-1852 had ~30 fold of reporter activity over the backbone pGL3-Basic vector, these results suggested that -1852~-1118 bp contained the important regulatory element in the Nanog promoter (**Figure 3**).

A potential effect identification of methylation and acetylation in Nanog promoter

To determine the contribution of the Nanog promoter methylation and acetylation to the expression of this gene, a common demethylating and histone deacetylase inhibitors agent, 5-Azadc and TSA was used. Because of being an analog of cytosine in DNA or cytidine in RNA, 5-Azadc, which is known as Vidaza®, titrates out the methyltransferase enzymes (DNMTs) and inhibits their functions by acting as a false substrate. Before the induction, we did the concentration screening of 5-Azadc and TSA. Panc-1 cells were transfected by pGL3-1852 and treated with different concentration of 5-Azadc and TSA. The reporter activity was the most highest when the concentration of 5-Azadc and TSA was 10 μmol/L and 1.0 μmol/L, respectively (**Figure 4A** and **4B**). Then, Panc-1 cells were transfected with pGL3-1852 and treated with 5-Azadc and TSA signally or together. It was clearly showed that the reporter activity was increased significantly when 5-Azadc and TSA was added together (**Figure 4C**), suggesting a possible role for methylation in regulating Nanog expression.

Meanwhile, Methyl specific primers (M) and unmethyl specific primers (U) were designed to test the methylation state. DNAs from Bxpc-3

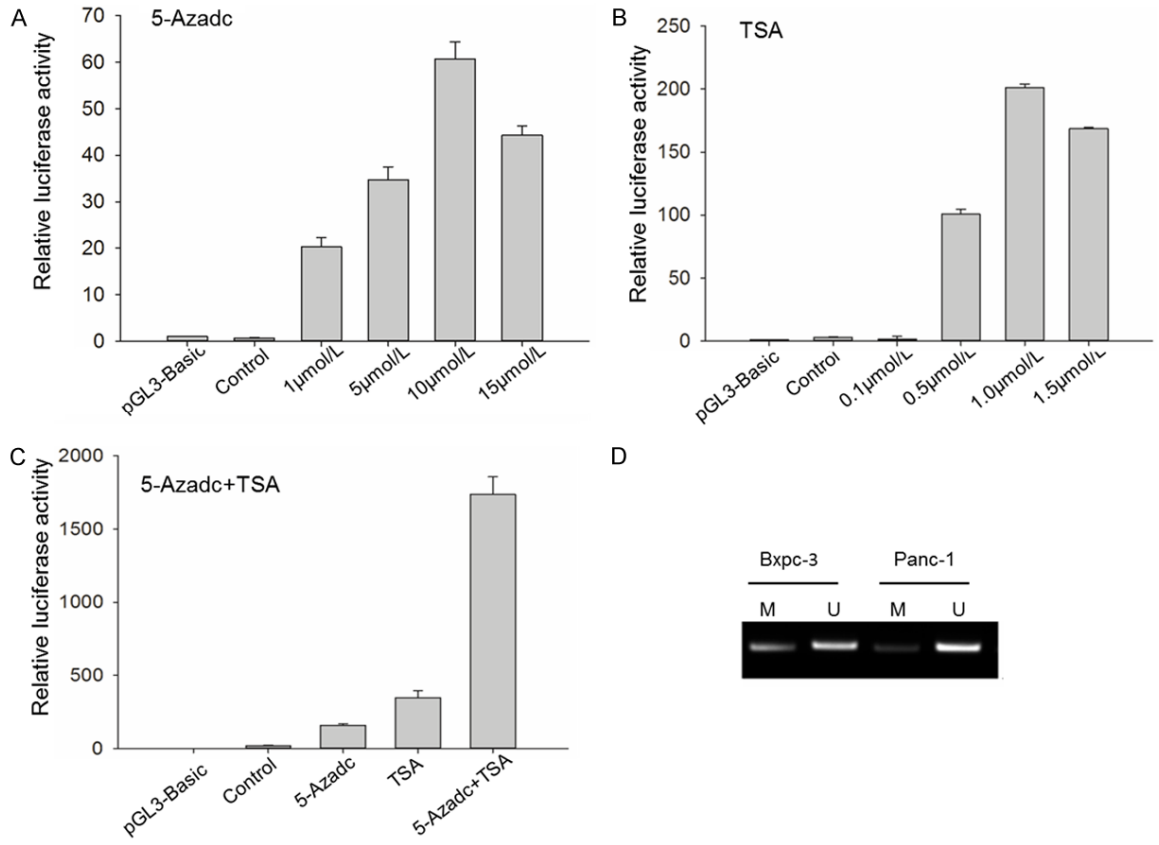

Figure 4. Effects of 5'azacytidine and in-vitro promoter methylation in Nanog expression. A and B. Panc-1 cells were transfected by pGL3-1852 and treated with different concentration of 5-Azadc and TSA. C. 5-Azadc and TSA was added together with the optimal concentration. D. Methyl specific primers (M) and unmethyl specific primers (U) were designed to test the methylation state.

(low expression of Nanog) and Panc-1 (high expression of Nanog) cells treated with bisulfite were amplified by PCR using two sets of primers. It is notable that amplification in Bxpc-3 cells was enhanced compared to Panc-1 cells (**Figure 4D**), suggesting the possibility of elevated methylation of the CpG island in the Nanog regulatory region in Bxpc-3 cells.

Discussion

Nanog protein is a transcription factor and contains a homeodomain, which plays a key role in embryonic stem cells. Nanog gene was found in 2003, a great of studies has confirmed its core position in the decision of the pluripotency of embryonic stem cells [3]. Nanog overexpression is a pluripotent marker in vitro and in vivo, and its low expression is an early marker of differentiation. In contrast, high expression of Nanog can compensate the artificial defects in ESC cell culture conditions. It is speculated that the level of Nanog expression may be the key conversion factors of cells between the state of differentiation and pluripotency [4].

NANOG is a transcription factor in embryonic stem cells (ESCs) and is thought to be a key factor in maintaining pluripotency. NANOG is thought to function in concert with other factors such as Oct-4 and SOX2 to establish ESC identity. These cells offer an important area of study because of their ability to maintain pluripotency. In other words, these cells have the ability to become virtually any cell of any of the three germ layers (endoderm, ectoderm, mesoderm). It is for this reason that understanding the mechanisms that maintain a cell's pluripotency is critical for researchers to understand how stem cells work; and may lead to future advances in treating degenerative diseases.

Overexpression of Nanog in mouse embryonic stem cells causes them to self-renew in the

absence of Leukemia inhibitory factor. In the absence of Nanog, mouse embryonic stem cells differentiate into visceral/parietal endoderm [5, 6]. Loss of Nanog function causes differentiation of mouse embryonic stem cells into other cell types [7]. NANOG overexpression in human embryonic stem cells enables their propagation for multiple passages during which the cells remain pluripotent [8]. Gene knockdown of Nanog promotes differentiation, thereby demonstrating a role for these factors in human embryonic stem cell self-renewal [9]. It has been shown that the tumor suppressor p53 binds to the promoter of NANOG and suppresses its expression after DNA damage in mouse embryonic stem cells. p53 can thus induce differentiation of embryonic stem cells into other cell types which undergo efficient p53-dependent cell-cycle arrest and apoptosis [10]. By using DNA microarray to find the transcription targets of Nanog, Nanog regulated genes have been identified. Some of these target genes explain the transformation of NIH3T3 cells [11]. Yamanaka et al., demonstrate induction of pluripotent stem cells from mouse embryonic or adult fibroblasts by introducing four factors, Oct3/4, Sox2, c-Myc, and Klf4, under ES cell culture conditions. Of these four factors it has been shown that Nanog was dispensable for such induction in this cell system [12].

More research has shown that the hematopoietic stem cells was transfected by Nanog gene and little effect on the cell self-renewal capacity, but lead to cell malignant tumor growth; therefore, Nanog was thought as a candidate cancer gene by some researchers [13]. But so far, there was little research about the expression of Nanog in pancreatic cancer cell lines, as well as its relationship with pancreatic cancer stem cell and function in maintaining pancreatic cancer, therefore, it is of great significance to explore its expression in pancreatic carcinoma.

This study also examined Nanog mRNA and protein level expression of three pancreatic cell lines with different differentiation degree. We found there was a certain amount of Nanog expression in those three cell line, the expression level of Nanog in Panc-1 and Mia PaCa-2 cell line was significantly higher than that of Bxpc-3 cell line, suggesting that Nanog may be associated with pancreatic cancer differentiation level. So, this study will bring a new hope for the research and treatment of pancreatic cancer about its expression and regulation mechanism, as well as the role in maintaining the tumor cell proliferation, differentiation and transfer.

Promoter activity of Nanog gene was investigated in this paper and the core regulatory regions was found at -1852-1112 bp. Methylation inhibitors and deacetylation inhibitors can significantly increase the Nanog gene promoter activity, suggesting that its high expression in tumor stem cells may be related with the epigenetic modification, then a thorough study on its relationship with the tumor stem cells will bring a new hope for the research and treatment of pancreatic cancer.

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Disclosure of conflict of interest

None

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